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(54) **METHOD OF IMPROVING DIASTOLIC DYSFUNCTION**

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(57) **ABSTRACT**

A method of treating, preventing, reversing, or ameliorating diastolic dysfunction includes reducing S-glutathionylated myosin binding protein-C (MyBP-C) level by administering to a host in need thereof a therapeutically effective amount of tetrahydrobiopterin (BH_4).

10 Claims, 17 Drawing Sheets

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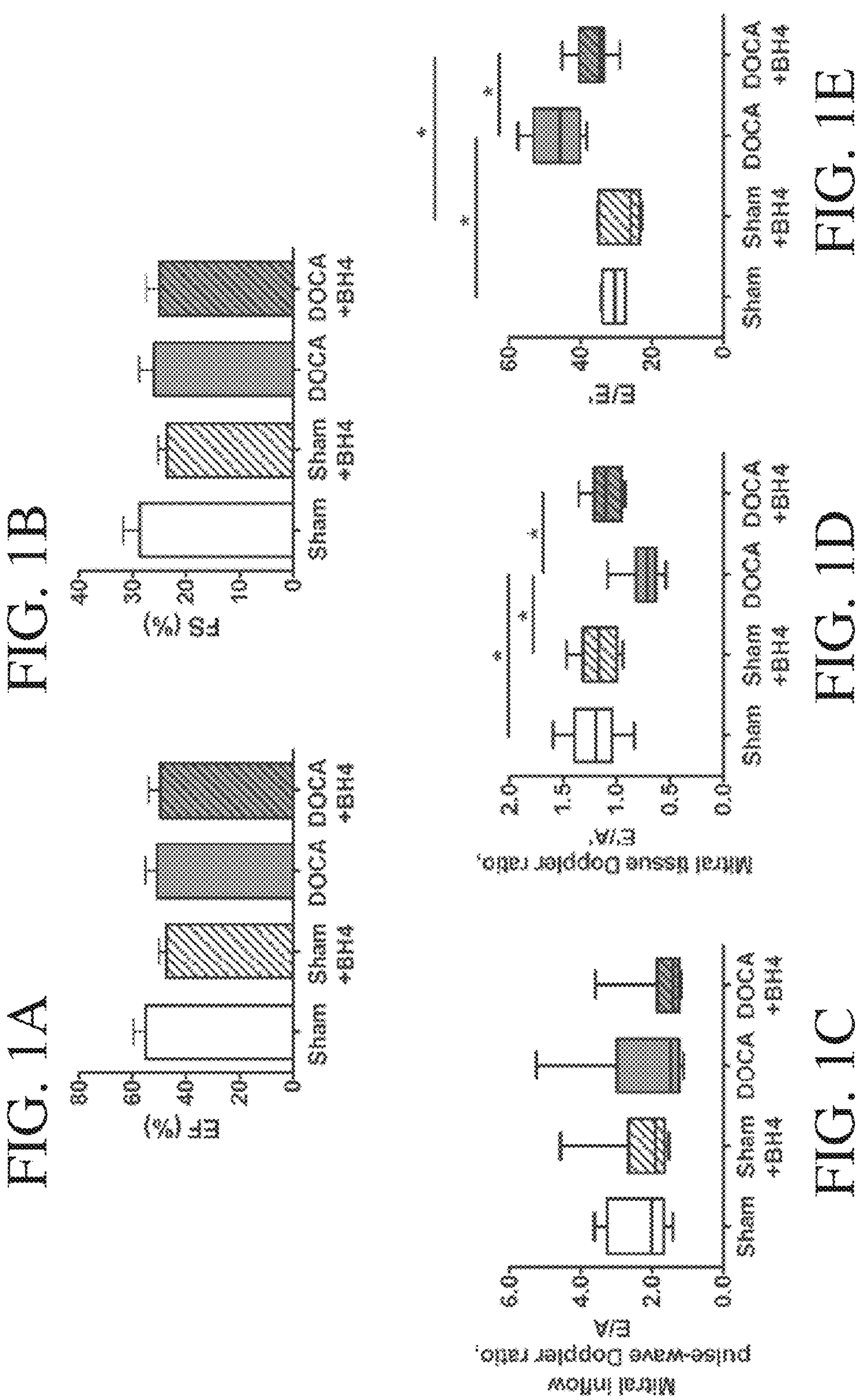


FIG. 1A

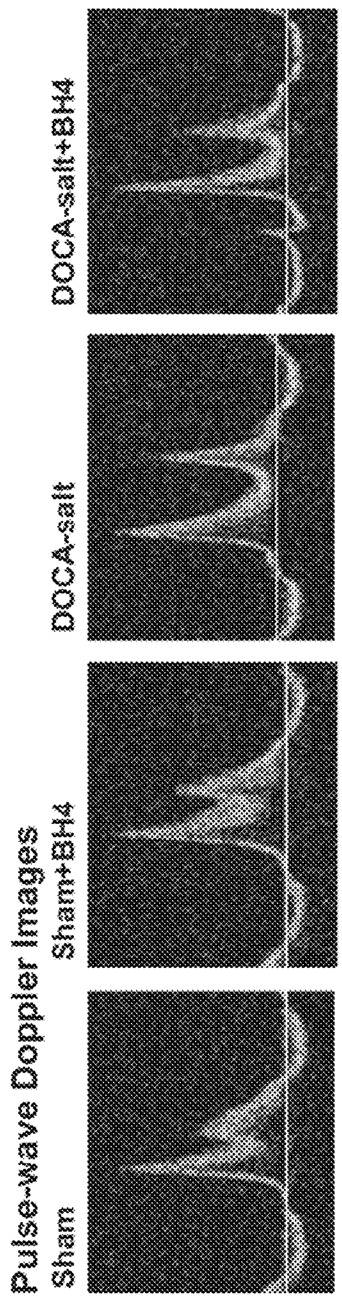
FIG. 1B

FIG. 1C

FIG. 1D

FIG. 1E

FIG. 1F


Pulse-wave Doppler Images
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Sham+BH4
DOCA-salt
DOCA-salt+BH4

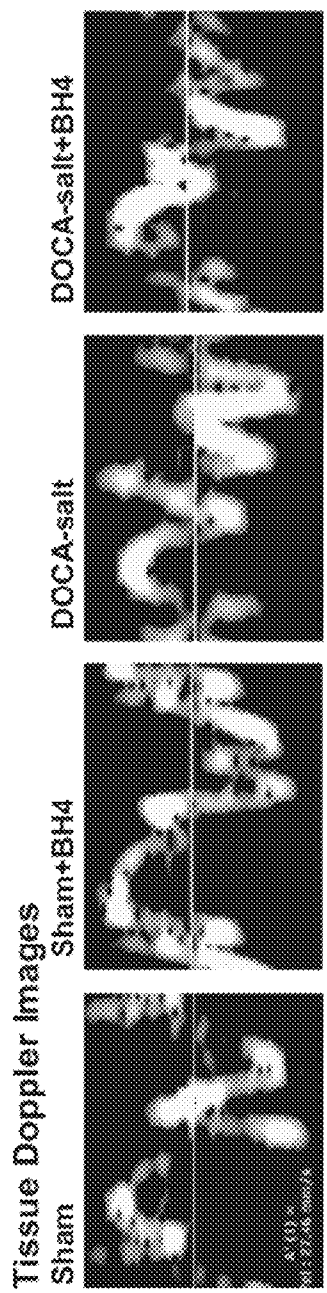
Tissue Doppler Images
Sham
Sham+BH4
DOCA-salt
DOCA-salt+BH4

FIG. 1G

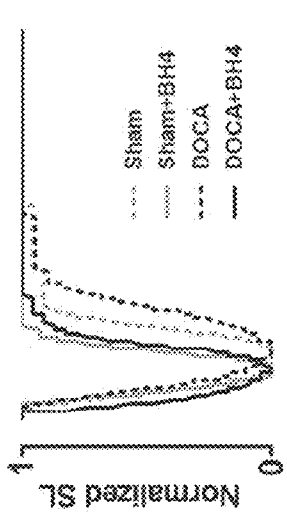

FIG. 2A

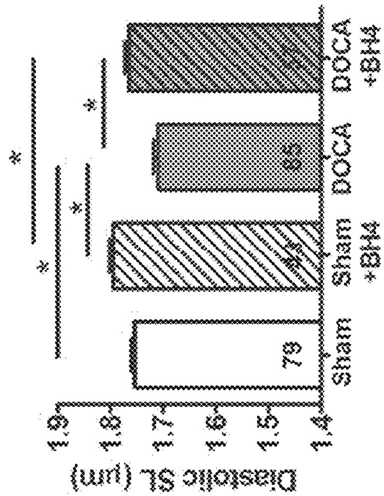

FIG. 2B

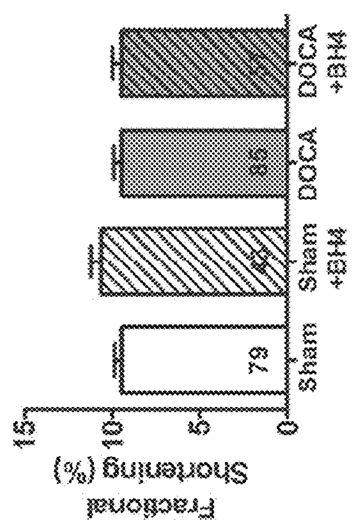

FIG. 2C

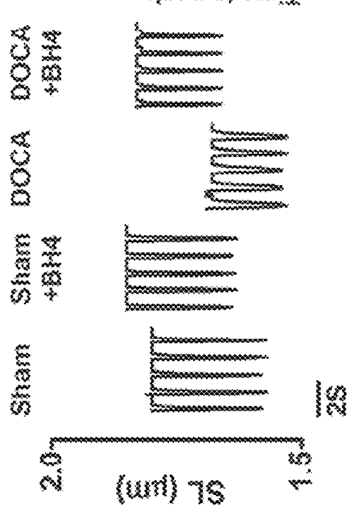

FIG. 2D

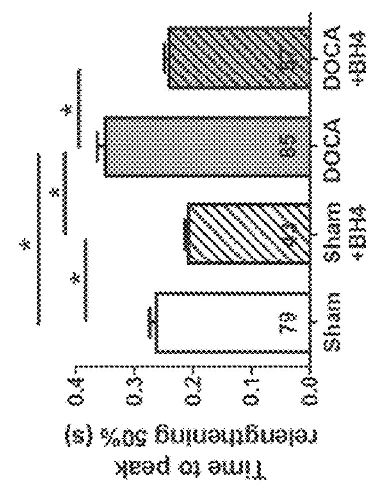

FIG. 2E

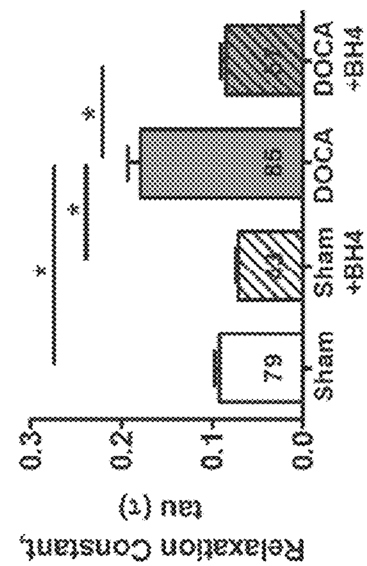

FIG. 2F

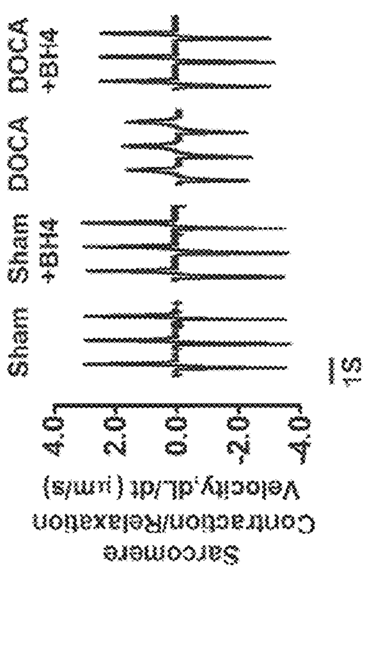

FIG. 2G

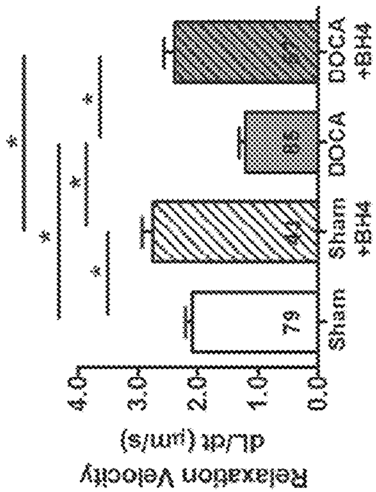

FIG. 2H

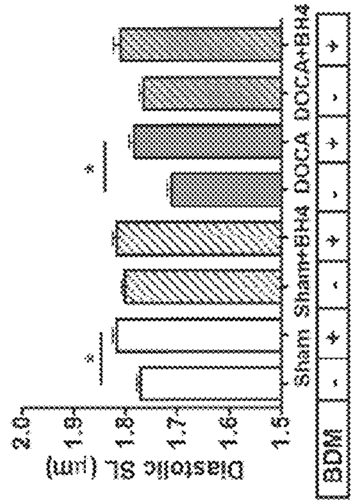

FIG. 2I

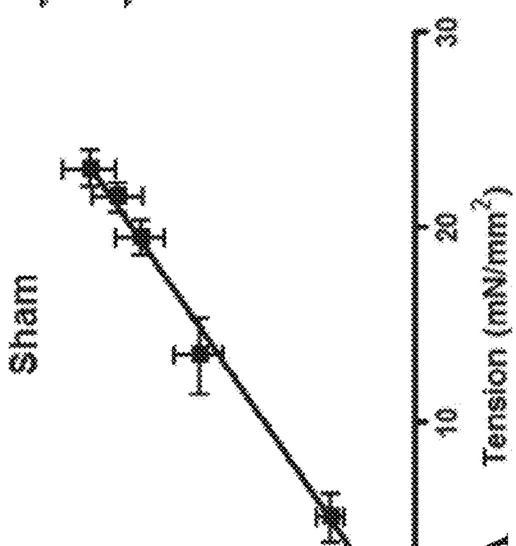

FIG. 3A

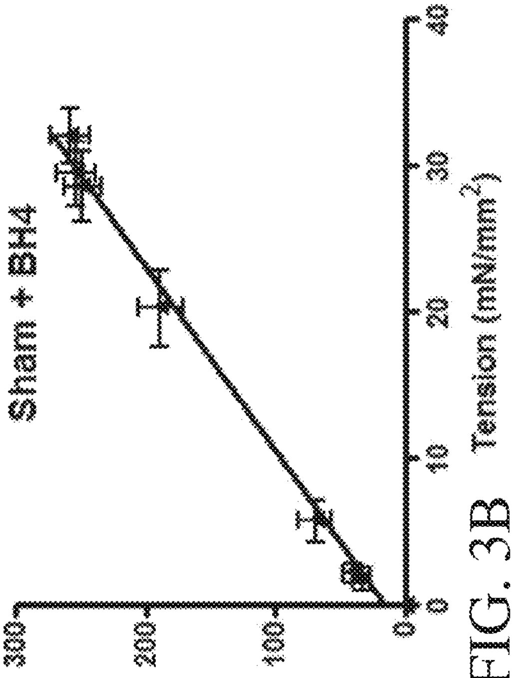

FIG. 3B

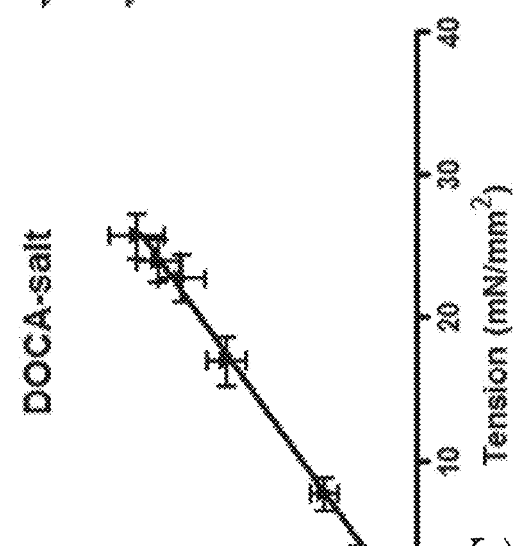

FIG. 3C

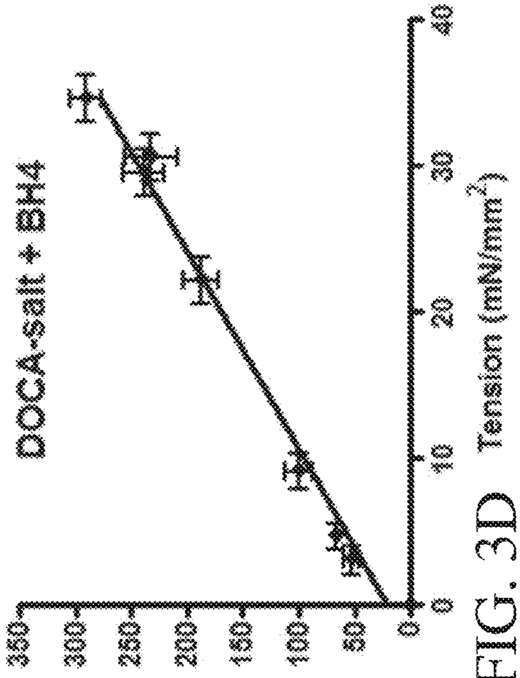

FIG. 3D

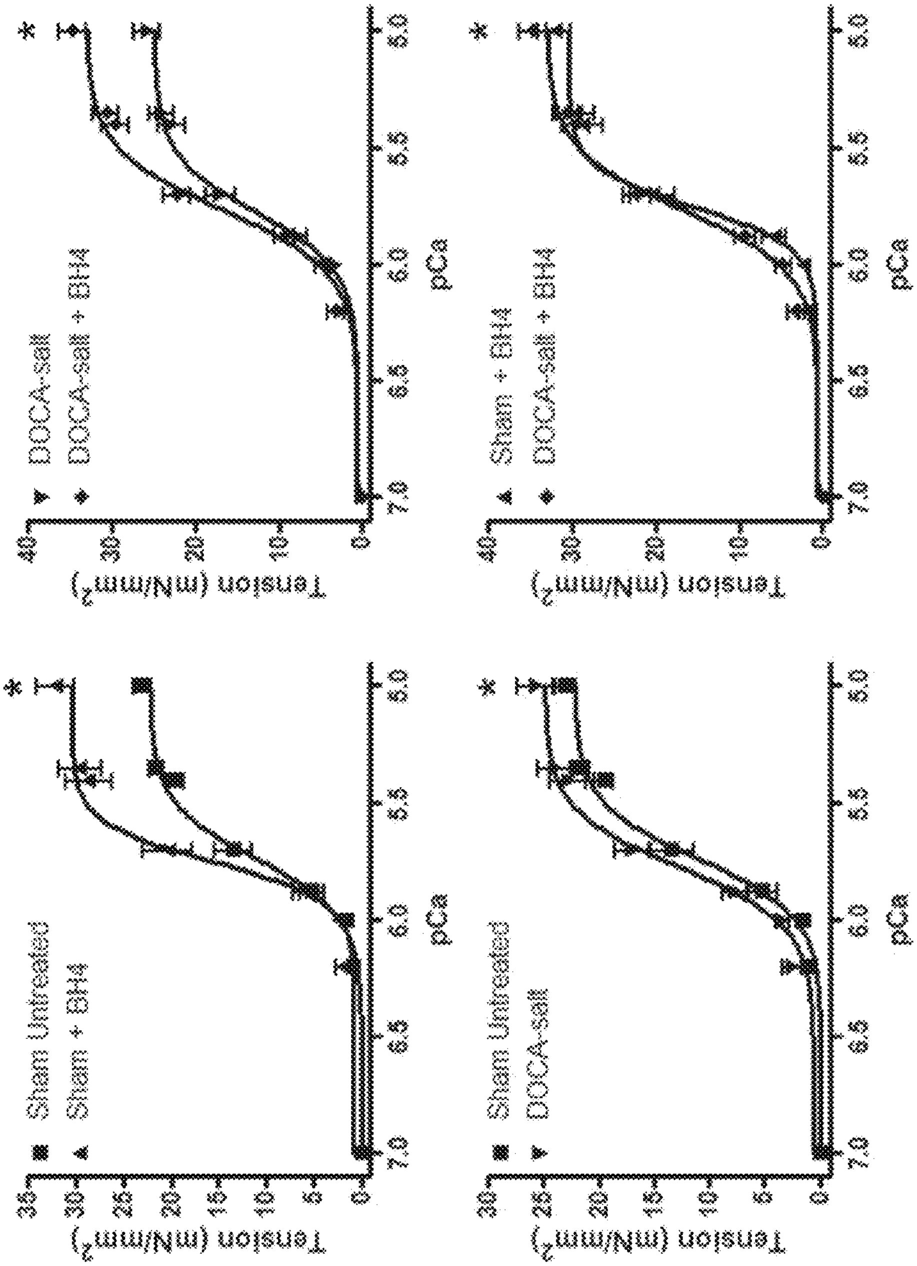
Sham Untreated
Sham + BH4
Tension (mN/mm2)
pCa
DOCA-salt
DOCA-salt + BH4
Sham + BH4
DOCA-salt + BH4
Sham Untreated
DOCA-salt
*

FIG. 4A

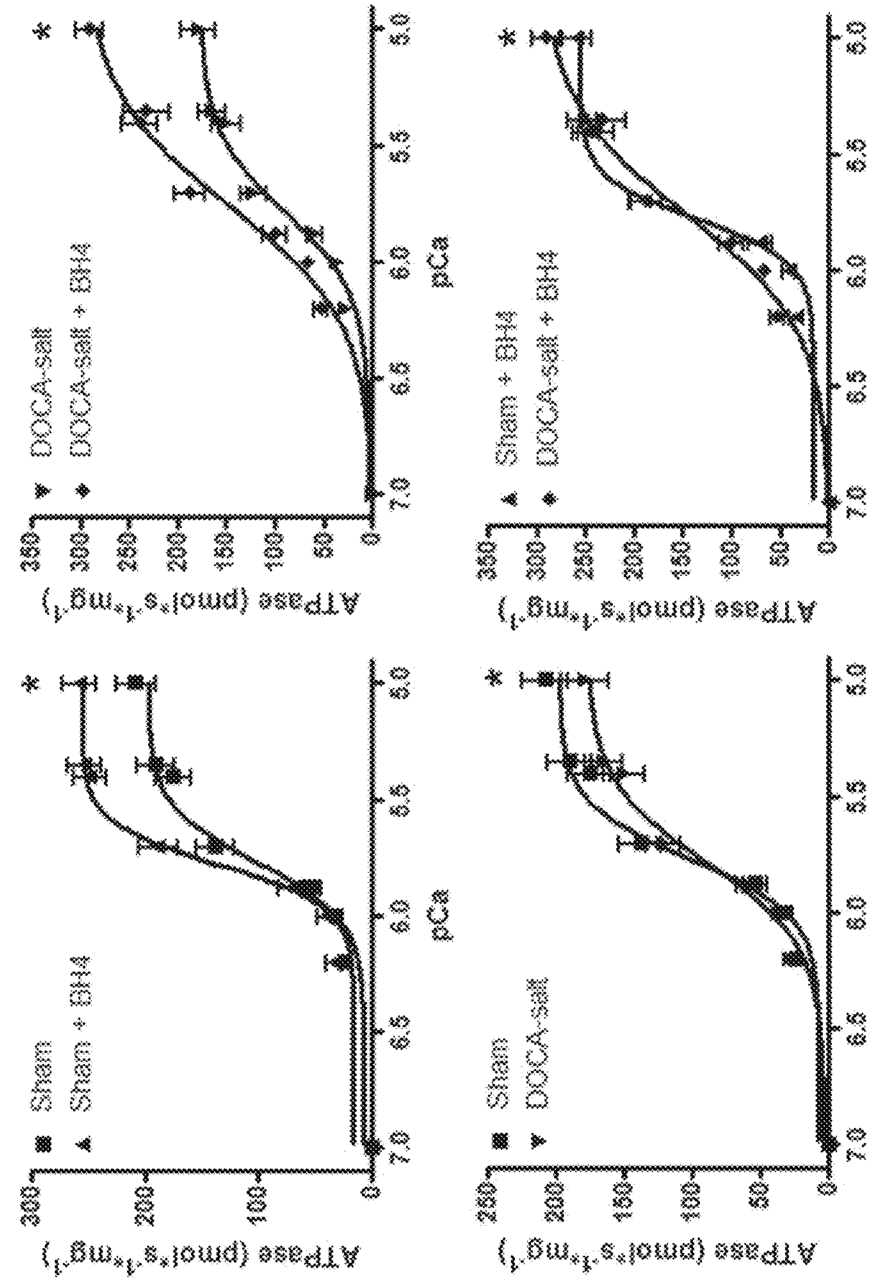
Sham
Sham + BH4
ATPase (pmol*s-1*mg-1)
pCa
DOCA-salt
DOCA-salt + BH4
Sham + BH4
*

FIG. 4B

FIG. 5A

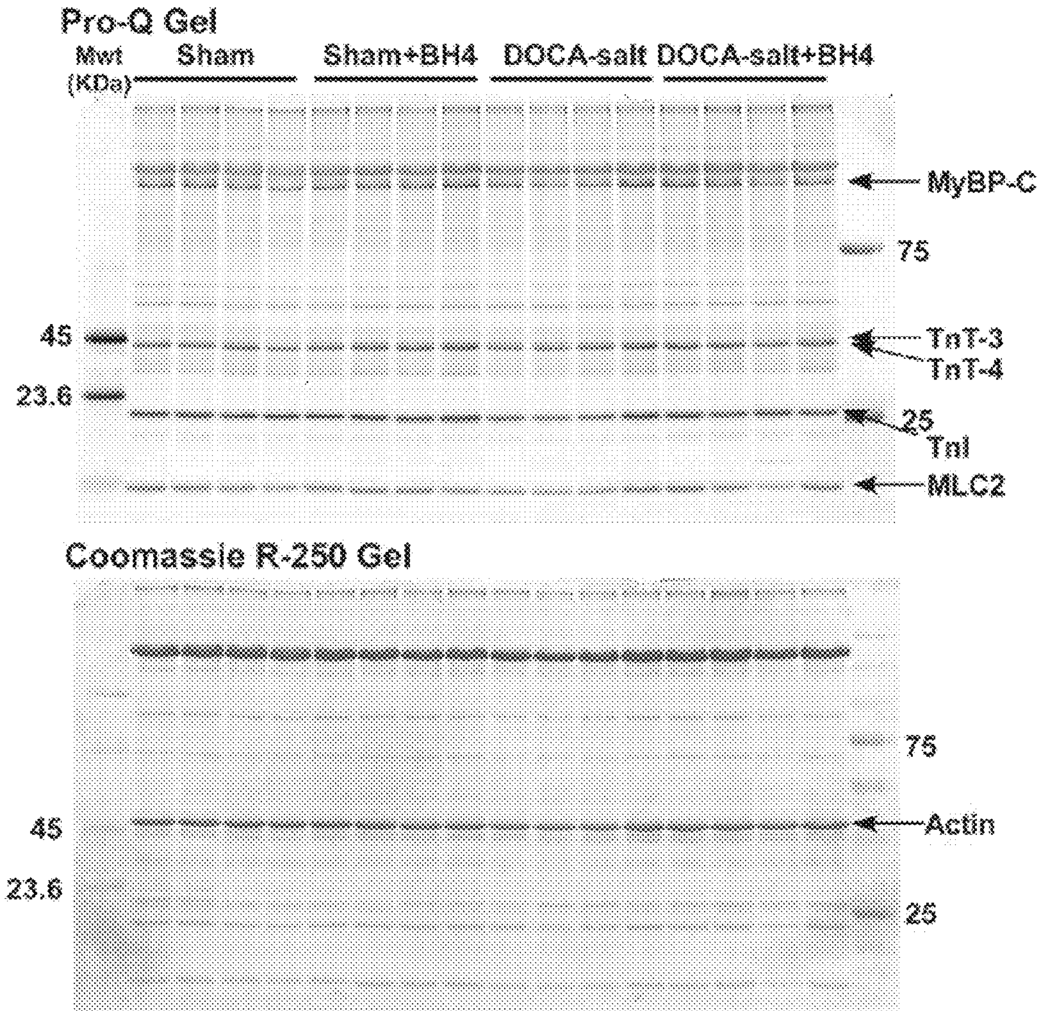
Pro-Q Gel
Mwt (KDa)
Sham
Sham+BH4
DOCA-salt
DOCA-salt+BH4
MyBP-C
75
45
TnT-3
TnT-4
23.6
25
TnI
MLC2
Coomassie R-250 Gel
75
45
Actin
23.6
25

FIG. 5B

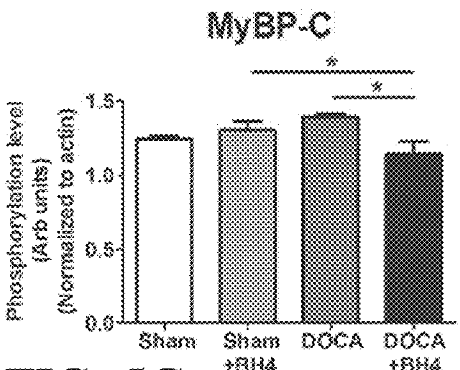

FIG. 5C

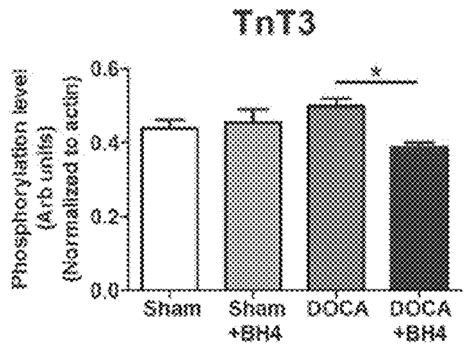

FIG. 5D

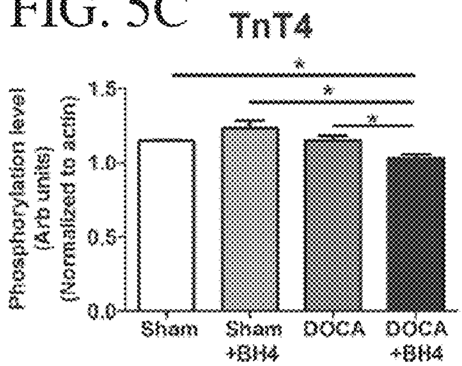

FIG. 5E

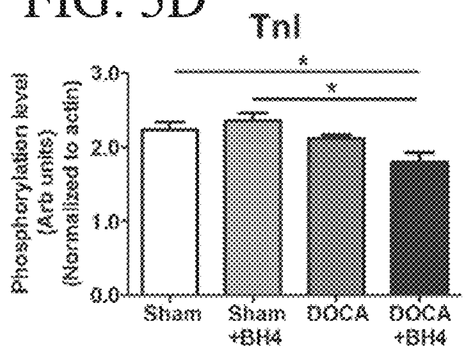

FIG. 5F

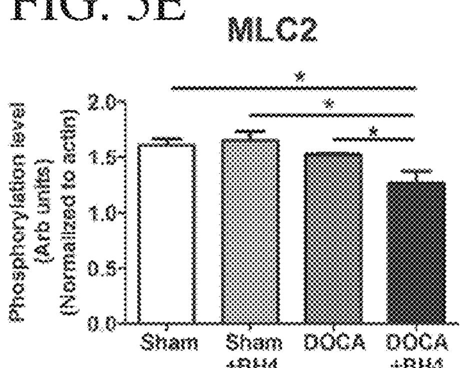

FIG. 5G

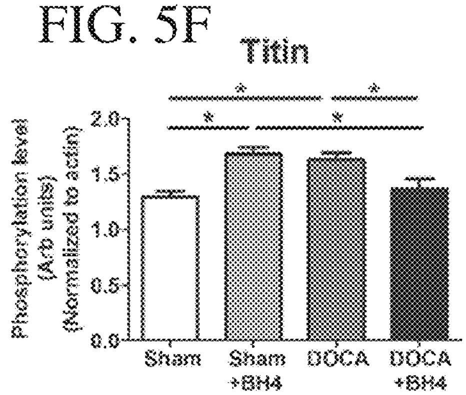

FIG. 5H

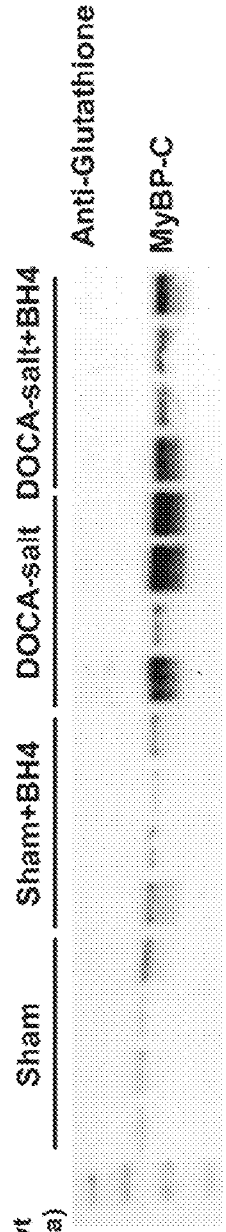

FIG. 6A

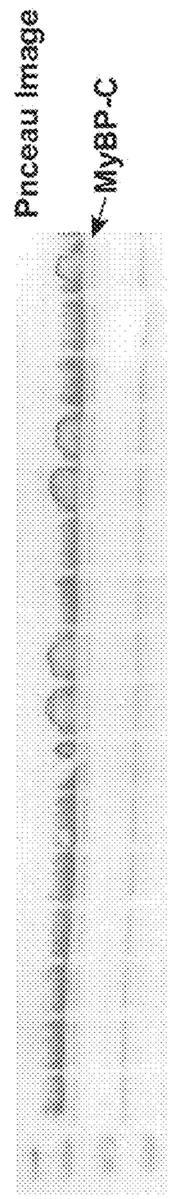

FIG. 6B

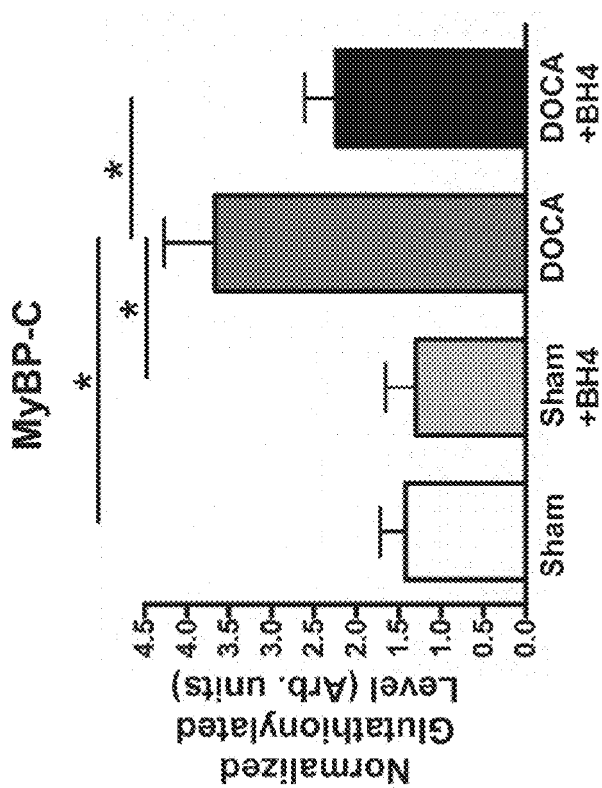

FIG. 6C

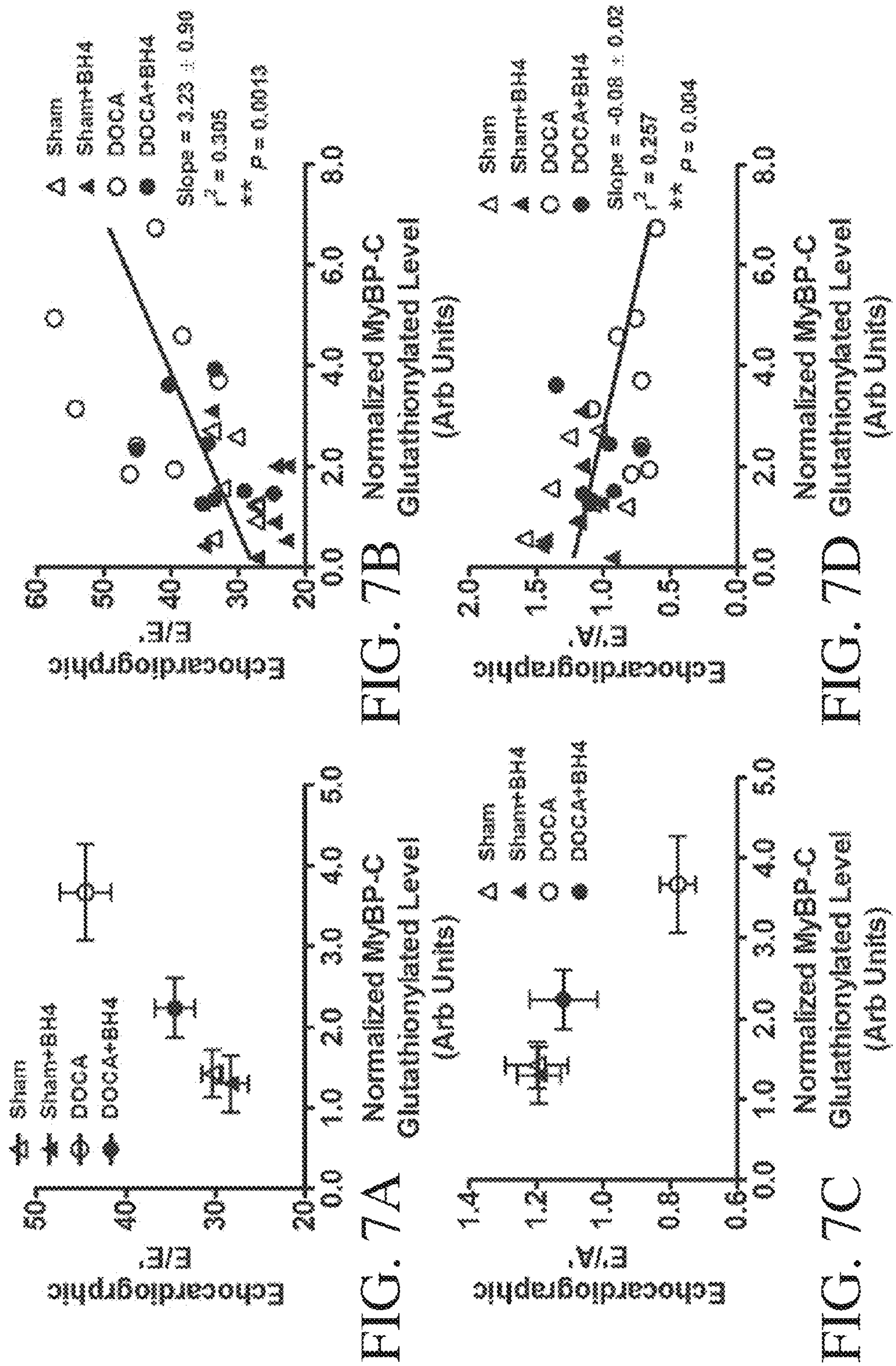

FIG. 7A

FIG. 7B

FIG. 7C

FIG. 7D

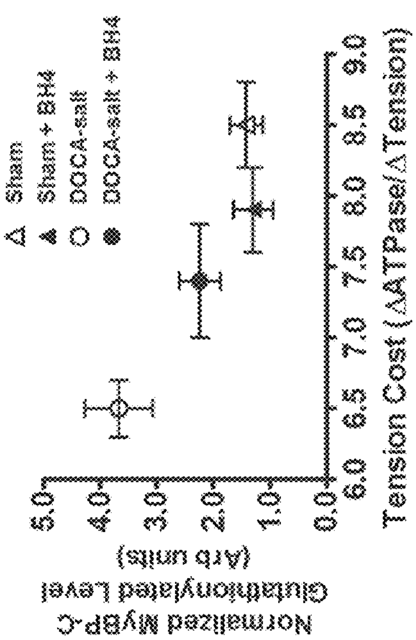

FIG. 7E

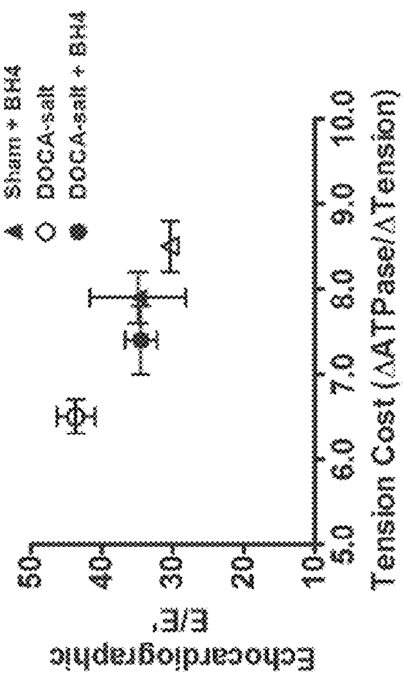

FIG. 7F

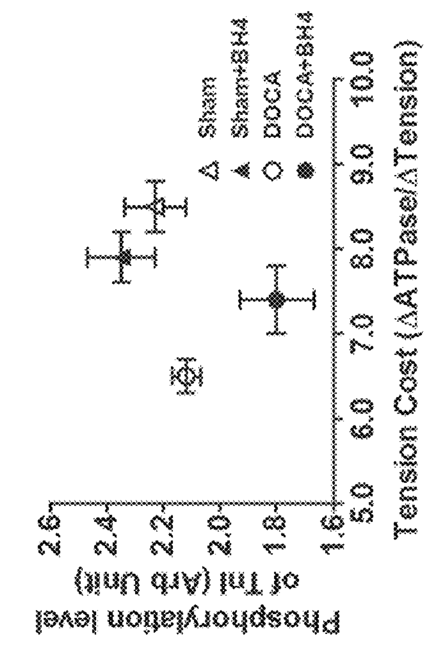

FIG. 7G

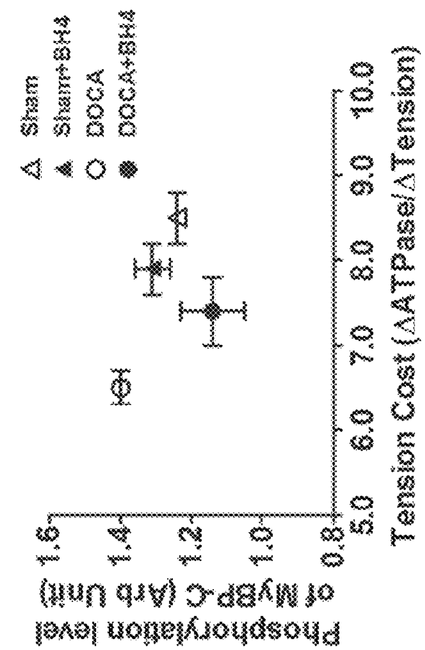

FIG. 7H

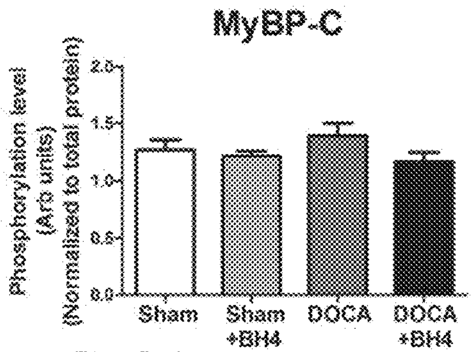

FIG. 8A

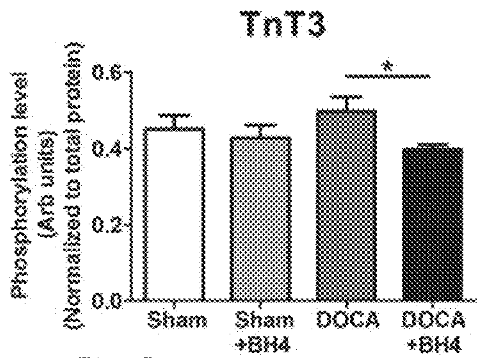

FIG. 8B

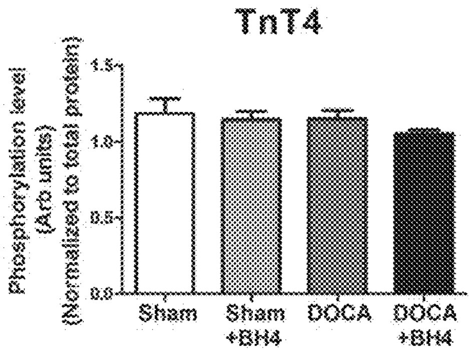

FIG. 8C

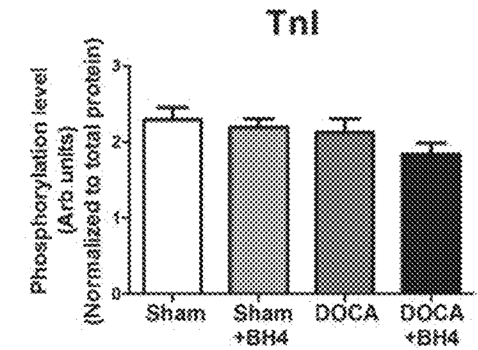

FIG. 8D

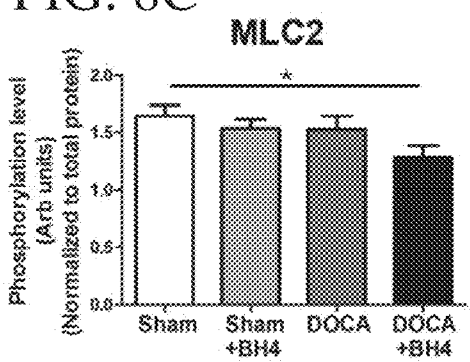

FIG. 8E

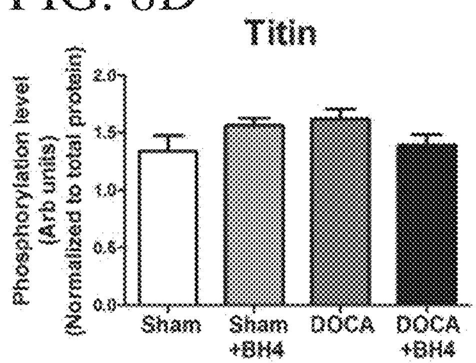

FIG. 8F

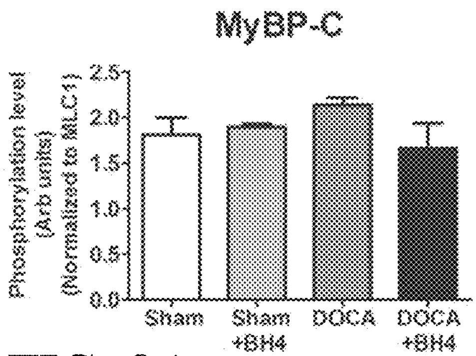

FIG. 9A

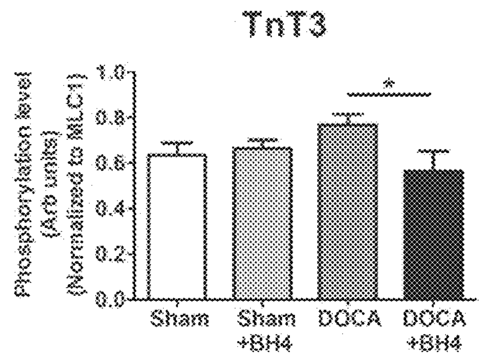

FIG. 9B

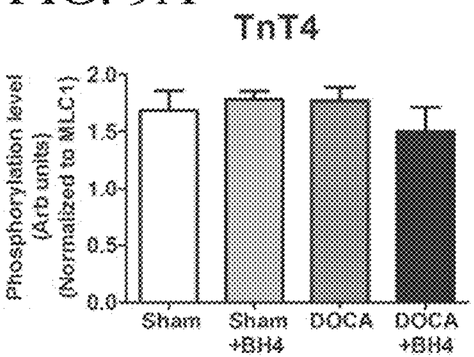

FIG. 9C

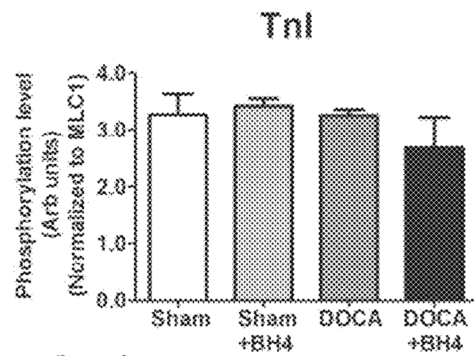

FIG. 9D

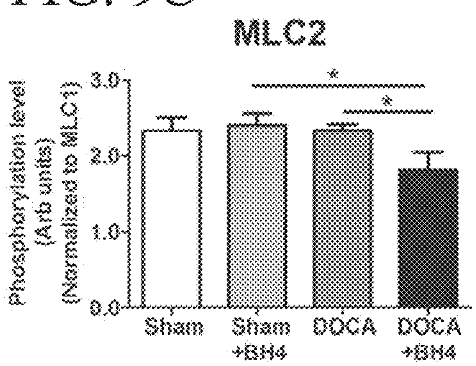

FIG. 9E

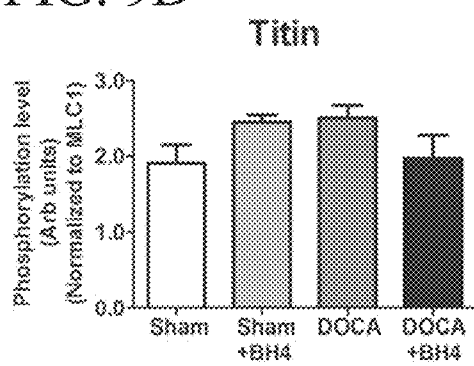

FIG. 9F

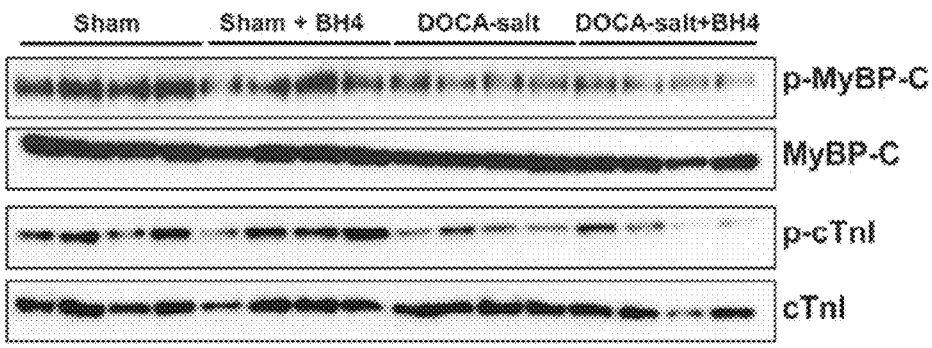

FIG. 10A

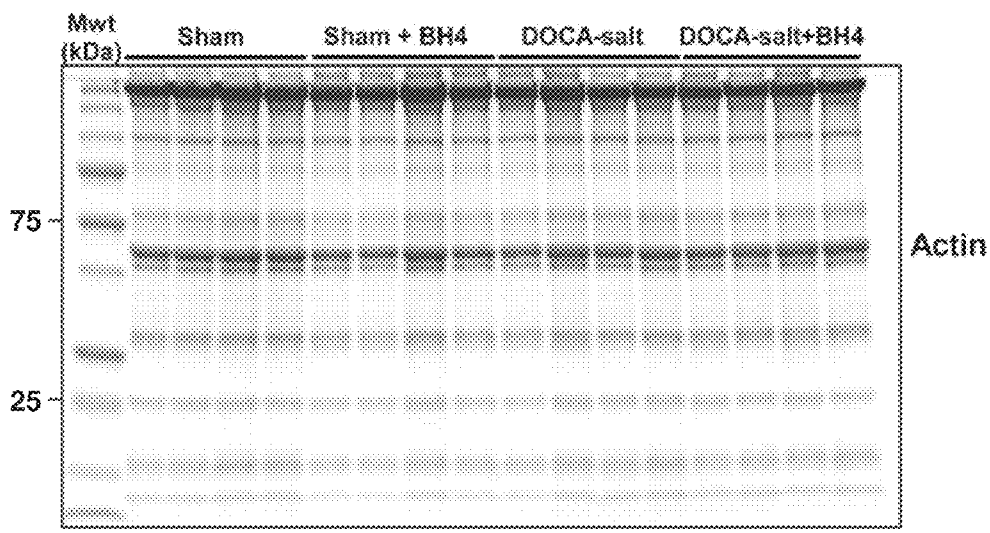

FIG. 10B

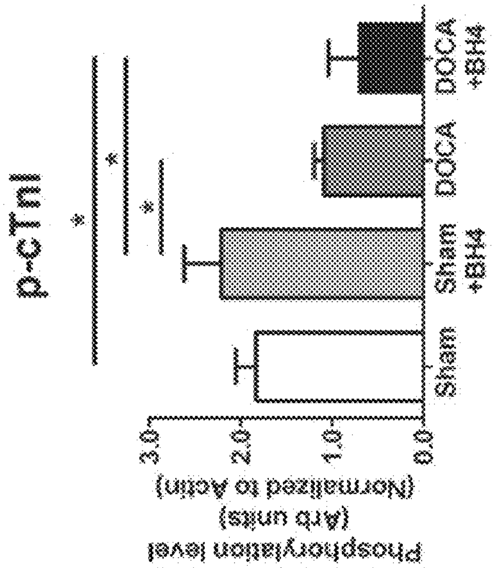

FIG. 10D

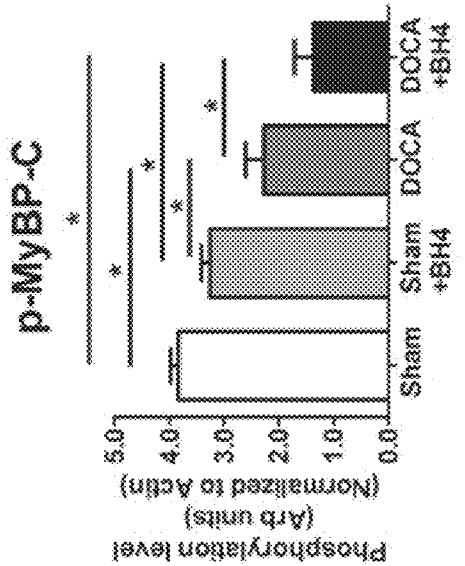

FIG. 10C

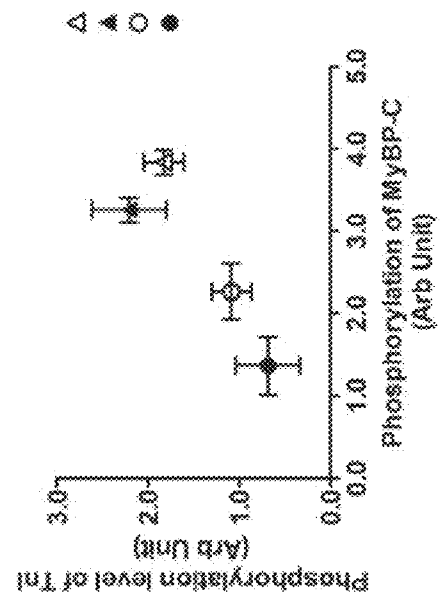

FIG. 10E

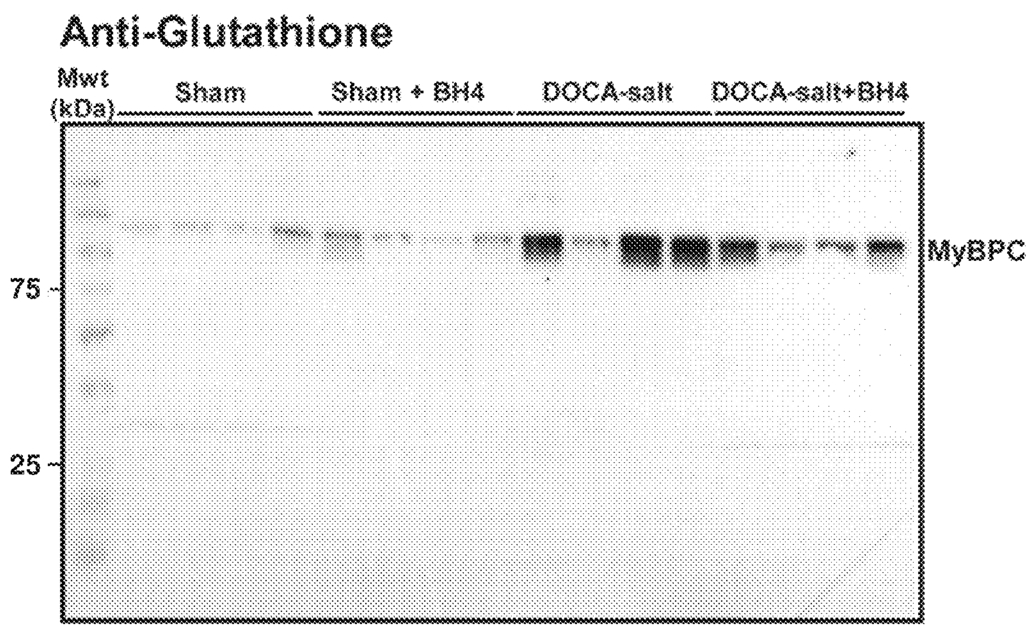
Anti-Glutathione
Mwt (kDa)
Sham
Sham + BH4
DOCA-salt
DOCA-salt+BH4
75
25
MyBPC

FIG. 11

METHOD OF IMPROVING DIASTOLIC DYSFUNCTION

CROSS-REFERENCE TO RELATED APPLICATIONS

This is a continuation-in-part (CIP) application of U.S. application Ser. No. 11/895,883, filed Aug. 27, 2007, which claims the priority benefit of U.S. Provisional Patent Application Ser. No. 60/840,368, filed Aug. 25, 2006, both are hereby incorporated herein in their entirety by reference. This application further claims the priority benefit of U.S. Provisional Patent Application Ser. No. 61/552,500, filed Oct. 28, 2011, which is also hereby incorporated herein in its entirety by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The work leading to the present invention was supported by NIH/NHLBI grants RO1 HL022231, RO1 HL064035, PO1 HL062426 to RJ S, and NIH/NHLBI grants RO1 HL085558, RO1 HL073753, PO1 HL058000, and a Veterans Affairs MERIT grant to SCD. MMM was supported by NIH T32 HL07692-16-20; DMT was supported by University of Illinois at Chicago Center for Clinical and Translational Science (Award Number UL1 RR029879) from the National Center for Research Resources, and by a University of Illinois at Chicago Fellowship. The U.S. Government therefore has certain rights in the invention.

FIELD AND BACKGROUND OF THE INVENTION

The present invention is generally directed to cardiac treatment and therapy, and more particularly to a method of treating, preventing, reversing, or ameliorating diastolic dysfuntion.

Hypertension is the most common risk factor for diastolic dysfunction in humans, which can lead to heart failure with preserved ejection fraction (Reference 1). This type of heart failure is increasing, and accounts for significant mortality and healthcare expenditures (References 1 and 2). Current treatments for diastolic dysfunction are inadequate, partially because the mechanism of altered myocardial relaxation is incompletely understood (Reference 3). Nitric oxide (NO) generated by NO synthase (NOS) is a critical modulator of cardiac relaxation (Reference 4), and NO bioavailability is regulated by tetrahydrobiopterin (BH_4) (Reference 5).

Under physiological conditions, NOS catalyzes the production of NO from L-arginine to modulate myofilament contractility through mechanisms that are not clear (References 6-9). BH_4 depletion, leads to NOS uncoupling (References 5and 10), the production of superoxide instead of NO, and diastolic dysfunction (References 5 and 11). BH_4 supplementation reverses these effects. Recently, I have reported that diastolic dysfunction was characterized by altered myofilament properties and by S-glutathionylation of cardiac myosin binding protein-C (MyBP-C) (Reference 12). S-glutathionylation is an oxidative post-translational modification of protein cysteines by the addition of the anti-oxidant tripeptide glutathione (References 13-15). I tested whether the improvement in diastolic dysfunction with BH_4 treatment correlated with changes in myofilament properties and in S-glutathionylation of cardiac MyBP-C.

ASPECTS OF THE INVENTION

The present disclosure is directed to various aspects of the present invention.

One aspect of the present invention is demonstration that by depressing or reducing S-glutathionylation of myosin binding protein-C (MyBP-C), tetrahydrobiopterin (BH_4) ameliorates diastolic dysfuntion by reversing a decrease in myofilament cross-bridge kinetics or restorating to normal thereof.

Another aspect of the present invention is demonstration of cardiac relaxation modulation by post-translational modification of myofilament proteins.

Another aspect of the present invention is demonstration that hypertension-induced diastolic dysfunction is characterized by reduced myofilament cross-bridge kinetics that are reversed by BH_4, and that the effect of BH_4 correlates with a reduction in glutathionylation of MyBP-C, suggesting that this post-translational modification may lead to diastolic dysfunction and that BH_4 treatment may work by preventing this oxidative modification.

Another aspect of the present invention is a method of treating, preventing, reversing, or ameliorating diastolic dysfunction, which includes reducing S-glutathionylated myosin binding protein-C (MyBP-C) level by administering to a host in need thereof a therapeutically effective amount of tetrahydrobiopterin (BH_4).

Another aspect of the present invention is a method of treating, preventing, reversing, or ameliorating diastolic dysfunction, which includes reversing changes in myofilament cross-bridge kinetics level by administering to a host in need thereof a therapeutically effective amount of tetrahydrobiopterin (BH_4).

Another aspect of the present invention is a method of treating, preventing, reversing, or ameliorating diastolic dysfunction, which includes restoring myofilament cross-bridge kinetics to normal level by administering to a host in need thereof a therapeutically effective amount of tetrahydrobiopterin (BH_4).

Another aspect of the present invention is a method of treating, preventing, reversing, or ameliorating diastolic dysfunction, which includes modulating post-translational modification of myosin binding protein-C (MyBP-C) level by administering to a host in need thereof a therapeutically effective amount of tetrahydrobiopterin (BH_4).

Another aspect of the present invention is a method of treating, preventing, reversing, or ameliorating diastolic dysfunction in a host with manganese superoxide dismutase (MnSOD) deficiency, which includes administering to the host a therapeutically effective amount of tetrahydrobiopterin (BH_4).

BRIEF DESCRIPTION OF THE DRAWINGS

One of the above and other aspects, novel features and advantages of the present invention will become apparent from the following detailed description of the non-limiting preferred embodiment(s) of invention, illustrated in the accompanying drawings, wherein:

FIGS. **1**A-G illustrate thoractic echocargiographic parameters in WT and DOCA-salt mice treated with or without BH_4. FIG. **1**A illustrates ejection fraction (%, EF) and FIG. **1**B illustrates fractional shortening (%, FS) determined in short axix M-mode view. FIG. **1**C illustrates mitral inflow pulse-wave Doppler ratio (E/A). FIG. **1**D illustrates mitral tissue doppler ratio, E'/A'. FIG. **1**E illustrates E/E'. FIGS. **1**F-G illustrate representative images from apical four chamber view of pulse-wave (F) and TDI (G). Data was represented mean±SEM. N=7-9 per group. Data were statistically analyzed using JMP statistical software by two-way ANOVA followed by Student's t-test. *P<0.05;

FIGS. 2A-I illustrate improved diastolic sarcomere length and relaxation impairement by BH_4 treatment. Isolated myocytes from sham, sham+BH_4, DOCA-salt and DOCA-salt+BH_4 groups were stimulated at 1 Hz recorded by Ionoptix. FIG. 2A illustrates normalized sarcomere trace. FIG. 2B illustrates diastolic resting SL of DOCA-salt group were restored by BH_4 treated group. FIG. 2C illustrates fractional shortening. FIG. 2D illustrates sarcomere contraction and relaxation trace. FIG. 2E illustrates peak 50% relengthening time. FIG. 2F illustrates relaxation constant, τ. FIG. 2G illustrates sarcomere contraction/relaxation velocity trace. FIG. 2H illustrates relaxation velocity. FIG. 2I illustrates BDM effect on sarcomere relaxations. BDM (10 mmole/L) were treated on isolated myocytes from Sham, Sham+BH_4, DOCA-salt, and DOCA-salt+BH_4 groups. DOCA-salt myocytes was increased residual SL by BDM, but there are no difference of residual SK between all groups after BDM treatment. Data was represented mean±SEM. Myocytes n number were indicated as accordingly from 5-7 mice per group. Data were statistically analyzed using JMP statistical software by two-way ANOVA followed by Student's t-test. *P<0.05;

FIGS. 3A-D illustrate tension cost for fibers. FIG. 3A illustrates tension cost for fibers from Sham. FIG. 3B illustrates tension cost for fibers from Sham+BH_4. FIG. 3C illustrates tension cost for fibers from DOCA-salt. FIG. 3D illustrates tension cost for fibers from DOCA-salt+BH_4 groups. Data was represented mean±SEM. N=9-17 fibers per group;

FIGS. 4A-B illustrate Ca^{2+}-sensitivity and ATPase of skinned fiber preparations. FIG. 4A illustrates maximal tension and pCa_{50} for tension are increased in fibers from DOCA-salt group compared to Sham group. FIG. 4B illustrates maximal ATPase increased in fibers from the DOCA-salt+BH_4 group compared to fibers from the DOCA-salt group. Data was represented mean±SEM. N=9-17 fibers per group. Data were statistically analyzed using JMP statistical software by two-way ANOVA followed by Student's t-test. *P<0.05;

FIGS. 5A-H illustrate phosphorylation levels of myofilaments proteins. FIG. 5A illustrates representative ProQ. FIG. 5B illustrates coomassie R-250 gel of skinned fiber myofibril proteins. FIG. 5C illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for MyBP-C. FIG. 5D illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for TnT3. FIG. 5E illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for TnT4. FIG. 5F illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for TnI. FIG. 5G illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for MLC2. FIG. 5H illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for titin. Data were normalized to actin and statistically analyzed using JMP statistical software by two-way ANOVA followed by Student's t-test. N=4 mice per group;

FIGS. 6A-C illustrate glutathionylation levels of MyBP-C. FIG. 6A illustrates representative Anti-Glutathione gel. FIG. 6B illustrates representative pnceau image. FIG. 6C illustrates MyBP-C glutathionylation level normalized to total lane. Band densitometry data were represented mean±SEM. N=8 mice per group. Data were statistically analyzed using JMP statistical software by two-way ANOVA followed by Student's t-test. *P<0.05; and

FIGS. 7A-H illustrate the relationship between MyBP-C glutathionylation, diastolic dysfunction, and tension cost. FIGS. 7A-B illustrate echocardiographic parameter-E/E' ratio was positively correlated with normalized MyBP-C glutathionylation level. FIGS. 7C-D illustrate echocardiographic parameter-E'/A' ratio was negatively correlated with normalized MyBP-C glutathionylation level. FIG. 7E illustrates tension cost vs. normalized MyBP-C glutathionylation level. FIG. 7F illustrates tension cost vs. echocardiographic E/E'-negatively correlated. FIGS. 7G-H illustrate tension cost vs. phosphorylation level of TnI (G) and phosphorylation level of MyBP-C(H) from ProQ data. N=7-8 mice per group. * indicates linear regression **P<0.01.

FIGS. 8A-F illustrate phosphorylation levels of myofilaments proteins normalized by total protein. FIG. 8A illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for MyBP-C. FIG. 8B illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for TnT3. FIG. 8C illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for TnT4. FIG. 8D illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for TnI. FIG. 8E illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for MLC2. FIG. 8F illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for titin. Data were normalized to total protein and statistically analyzed using JMP statistical software by two-way ANOVA followed by Student's t-test. N=4 mice per group.

FIGS. 9A-F illustrate phosphorylation levels of myofilaments proteins normalized by MLC1. FIG. 9A illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for MyBP-C. FIG. 9B illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for TnT3. FIG. 9C illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for TnT4. FIG. 9D illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for TnI. FIG. 9E illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for MLC2. FIG. 9F illustrates phosphorylation levels of myofilament proteins as assessed by ProQ for titin. Data were normalized to MLC1 and statistically analyzed using JMP statistical software by two-way ANOVA followed by Student's t-test. N=4 mice per group.

FIG. 10A-E illustrate phosphorylation levels of MyBP-C and cTnI. FIG. 10A illustrates phosphorylation levels of myofilament proteins as assessed by Western blotting against specific antibodies, Phospho-ser282-MyBP-C, MyBP-C, phospho-Ser23/24-cTnI, and cTnI. FIG. 10B illustrates SDS-PAGE. Densitometry of Western blotting using phospho-Ser282-MyBP-C. FIG. 10C illustrates phospho-Ser23/24-TnI. FIG. 10D illustrates normalized to actin. FIG. 10E illustrates phosphorylation levels from TnI and MyBP-C were correlated. Data were normalized to actin and statistically analyzed using JMP statistical software by two-way ANOVA followed by Student's t-test. N=4 mice per group.

FIG. 11 illustrates glutathionylation levels of MyBP-C. Whole blot image of MyBP-C glutathionylation level against anti-glutathione antibody.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT(S) OF THE INVENTION

A few preferred embodiments of the present invention are described in detail sufficient for one skilled in the art to practice the present invention. It is understood, however, that the fact that a limited number of preferred embodiments are described herein does not in any way limit the scope of the present invention.

Recently, I reported that hypertension-induced diastolic dysfunction was accompanied by cardiac BH_4 depletion, NOS uncoupling, a depression in myofilament cross-bridge kinetics, and S-glutathionylation of myosin binding protein C

(MyBP-C). I hypothesized that the mechanism by which BH_4 ameliorates diastolic dysfunction is by preventing glutathionylation of MyBP-C and thus reversing changes of myofilament properties that occur during diastolic dysfunction. I used the deoxycorticosterone acetate (DOCA)-salt mouse model, which demonstrates mild hypertension, myocardial oxidative stress, and diastolic dysfunction.

As noted in more detail below, the mice were divided into two groups that received control diet and two groups that received BH_4 supplement for 7 days after developing diastolic dysfunction at post-operative day 11. Mice were assessed by echocardiography. Left ventricular papillary detergent-extracted fiber bundles were isolated for simultaneous determination of force and ATPase activity. Sarcomeric protein glutathionylation was assessed by immunoblotting. DOCA-salt mice exhibited diastolic dysfunction that was reversed after BH_4 treatment. Diastolic sarcomere length (DOCA-salt 1.70±0.01 vs. DOCA-salt+BH_4 1.77±0.01 μm, P<0.001) and relengthening (relaxation constant, τ, DOCA-salt 0.28±0.02 vs. DOCA-salt+BH_4 0.08±0.01, P<0.001) were also restored to control by BH_4 treatment. pCa_{50} for tension increased in DOCA-salt compared to sham, but reverted to sham levels after BH_4 treatment. Maximum ATPase rate and tension cost (ΔATPase/ΔTension) decreased in DOCA-salt compared to sham, but increased after BH_4 treatment. Cardiac MyBP-C glutathionylation increased in DOCA-salt compared to sham, but decreased with BH_4 treatment. MyBP-C glutathionylation correlated with the presence of diastolic dysfunction.

My results herein suggest that by depressing S-glutathionylation of MyBP-C, BH_4 ameliorates diastolic dysfunction by reversing a decrease in cross-bridge turnover kinetics. These data provide evidence for modulation of cardiac relaxation by post-translational modification of myofilament proteins.

Here, I demonstrate that oral administration of BH_4 improves diastolic dysfunction, reverses the changes in actin-myosin cross-bridge cycling, and decreases S-glutathionylated MyBP-C. My results support the hypothesis that oxidative post-translational modifications and associated modulation of myofilament properties is a molecular mechanism for diastolic dysfunction.

Methods

All protocols were in accordance with the guidelines of the Animal Care and Use Committee of the University of Illinois and comply with the laws of the United States of America.

EXAMPLE I

Generation of DOCA-Salt Mouse Model

Previously, I have shown that the DOCA-salt mouse model leads to mild hypertension, NOS uncoupling, myocardial oxidative stress, and diastolic dysfunction (Reference 10). A gradual and mild elevation in blood pressure was induced by unilateral nephrectomy, subcutaneous implantation of a controlled release deoxycorticosterone acetate (DOCA) pellet (0.7 mg/d; Innovative Research of America, Sarasota, Fla.), and substituting drinking water with 1.05% saline. Control animals underwent a sham operation, had placebo pellet implantation, and received water without salt.

Administration of BH_4

Mice were divided into two groups which received a control diet (sham N=7; DOCA-salt N=10) and two groups which received a BH_4 supplemental diet of 5 mg BH_4/day (Research Diets Inc, New Brunswick, N.J.; sham+BH_4 N=8; DOCA-salt+BH_4 N=8). The supplemental diet began on day 11 after surgery, and continued until day 18, when the mice were analyzed and sacrificed.

Transthoracic Echocardiography

Mitral pulse wave Doppler flow and tissue Doppler imaging (TDI) were performed using the Vevo 770 high-resolution in vivo imaging system (Visual Sonics, Toronto, Canada) (Reference 10). Mice were anesthetized with 1-1.5% isoflurane until a heart rate of around 350-390 beats/min was achieved because measures of diastolic function are sensitive to heart rate and loading conditions. M-mode images in the parasternal long axis and the left ventricle (LV) short-axis views at the mid-papillary level were taken. Measurements were averaged from five consecutive beats during expiration. The images for each mouse were recorded for at least 5 s (30-40 cardiac cycles) from which three to five representative cycles with the highest quality imaging were selected. Percent fractional shortening (% FS) was calculated as 100×(LVEDd)−(LVESd)/(LVEDd) and percent LV ejection fraction (% EF) was calculated as $100\times[(7/2.4+\text{LVEDd})\times \text{LEDd}^3]-[(7/2.4+\text{LVESd})\times \text{LVESd}^3]/[(7/2.4+\text{LVEDd})\times \text{LEDd}^3]$. Doppler measurements were made at the tips of the mitral leaflets for diastolic filling profiles in the apical four-chamber view. Mitral inflow velocities, peak early (E) and late (A) were measured by conventional pulsed-wave Doppler. TDI was used to determine the mitral annulus longitudinal velocities (Sm, E', and A') (Reference 10). Baseline images before treatment were acquired to confirm diastolic dysfunction in DOCA-salt mice. Subsequently, the mice were fed with BH_4, followed by echocardiography at day 18.

Cardiomyocyte Studies

Ventricular myocytes were isolated as previously described (Reference 10). Hearts were excised from anesthetized mice, perfused with buffer (in mmol/L: NaCl 113, KCl 4.7, Na_2HPO_4 0.6, KH_2PO_4 0.6, $MgSO_4$ 1.2, Phenol Red 0.032, $NaHCO_3$ 12, $KHCO_3$ 10, HEPES 10, Taurine 30, 2-3-butanedione monoxime 10) and digested with collagenase II (Worthington Biochemical Co. Lakewood, N.J.) for 7-8 min with 37° C. perfusion. Cardiomyocytes were washed with control buffers (in mmol/L: NaCl 133.5, KCl 4, Na_2HPO_4 1.2, HEPES 10, $MgSO_4$ 1.2 and 0.1% Bovine serum albumin) with serially increasing Ca^{2+} concentrations (0.2, 0.5, and 1 mmol/L). Then, myocytes were maintained in MEM medium (modified Eagle's medium with 1% insulin-transferrin-selenium, 0.1% bovine serum albumin, 1% glucose, and 1% penicillin/streptomycin) in a 95% O_2/5% CO_2 incubator at 37° C. until use.

The mechanical properties of the cardiomyocytes were assessed using an IonOptix Myocam System (IonOptix Inc., Milton, Mass.) as described previously (Reference 12). Unloaded cardiomyocytes isolated from each group of mice were placed on a glass slide and allowed to adhere for 5 min, then imaged with an inverted microscope and perfused with a normal Tyrode's buffer (in mmol/L: 133 NaCl, 5.4 KCl, 5.3 $MgCl_2$, 0.3 Na_2PO_4, 20 HEPES, 10 glucose, pH 7.4) containing 1.2 mmol/L calcium at 37° C. with a temperature controller. Cardiomyocytes were paced with 10 V, 4 ms square wave pulses at 1.0 Hz, and sarcomere shortening and relengthening were assessed using the following indices: diastolic sarcomere length (SL), peak fractional shortening (FS, %), the prolonged relaxation time constant $\tau(a_0+a_1e^{t/\tau}$, t=time), relengthening time (s), and maximum relaxation velocity (dL/dt).

2,3-Butanedione monoxime (BDM), a cross-bridge inhibitor, was used to measure residual sarcomere length. BDM inhibits the Ca^{2+} regulated attachment of the cross-bridges and force-generation of the attached cross-bridges (Reference 16). Isolated single myocytes were loaded on an chamber and perfused with BDM (10 mM) in Tyrode's solution at 37° C. Sarcomere length was again measured while the myocytes were field-stimulated as described above.

Dissection of Left Ventricular Papillary Muscles and Preparation of Skinned Fibers

Mice were anesthetized with pentobarbital (50 mg/kg IP), and the hearts were rapidly excised and rinsed in ice-cold relaxing solution (pH 7.0) composed of (in mM) 10 EGTA, 41.89 K-Prop, 6.57 $MgCl_2$, 100 BES, 6.22 ATP, 5 Na azide, and 10 creatine phosphate. The solution also contained 1 μg/mL leupeptin, 2.5 μg/ml pepstatin A, and 50 μM phenylmethylsulfonyl fluoride. Left ventricular papillary muscles were dissected and fiber bundles were prepared as previously described (Reference 17). The fiber bundles were extracted overnight in relaxing solution plus 1% Triton X-100 at 4° C.

Simultaneous Determination of Force and ATPase Activity in Detergent-Extracted Cardiac Fiber Bundles

Force and ATPase rate were measured simultaneously as previously described (Reference 17) using an experimental apparatus also previously described (Reference 18). The fiber bundles were mounted between a force transducer and displacement motor using aluminum T-clips, and the sarcomere length was set to 2.2 μm using He—Ne laser diffraction (Reference 19). The width and diameter were each measured at three points along the fiber bundle. Force per cross-sectional area was used to determine tension. The fiber was initially contracted at a saturating calcium concentration (pCa 4.5) and sarcomere length was again adjusted to 2.2 μm. Sarcomere length remained constant throughout the rest of the experiment.

ATPase activity was measured at 20° C. as previously described (References 17 and 20) and calibrated with rapid injections of ADP (0.5 nmol) with a motor-controlled syringe. The fiber was placed in relaxing solution for 2 min, then in the pre-activation solution for 2-3 min each time before being placed in the activating solution for 1-2 min (until stabilization of force) and then quickly returned to the relaxing solution. Various contraction-relaxation cycles were carried out using different ratios of total calcium concentration to total EGTA concentration. The final contraction was again at pCa 4.5.

Analysis of Sarcomeric Protein Phosphorylation

In one series of experiments, I employed Pro-Q Diamond (Invitrogen) gel stain to determine changes in phosphorylation of myofilament proteins. I also employed site specific antibodies for MyBP-C (anti-phospho-peptide-Ser282) and for cTnI (anti-phopho-Ser23/Ser24). Detailed methods are presented below.

Analysis of Sarcomeric Protein Glutathionylation by Western Immunoblotting

Myofibrils were prepared from DOCA-salt and sham model hearts, and pellets were solubilized in a non-reducing 2× Laemmli buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris HCl pH 6.8). 25 mM N-ethylmaleimide (NEM) was added to the standard rigor buffer with Triton X-100, the standard rigor wash buffer and the 2× Laemmli buffer. (Reference 21). Using the protein concentration determined from an RC-DC (Bio-Rad) assay, 40 pg of total protein was applied to a 12% SDS-PAGE gel and transferred onto a 0.2 μm PVDF membrane. The blot was blocked in 5% nonfat dry milk with 2.5 mM NEM for 1 h. Anti-glutathione mouse monoclonal primary antibody (Virogen) was used at 1:1000 dilution along with anti-mouse HRP-conjugated secondary antibody (Sigma) at 1:100,000 dilution to detect for S-glutathionylation (Reference 22). Optical density of the bands was measured with ImageQuant TL (GE Healthcare) and exported to Excel for further analysis.

Statistical Analysis

Echocardiography, sarcomere shortening, skinned fiber tension, and ATPase measurements, as well as post-translational modifications of myofilament proteins, were statistically analyzed by two-way ANOVA followed by student's t test using JMP statistical software. Analysis of the relation between Ca^{2+} and tension or ATPase activity was fitted using a modified Hill equation as described previously [20]. Analysis of the relation between MyBP-C glutathionylation and echocardiographic, E/E' ratio was correlated in linear regression analysis. A value of $P<0.05$ was considered significantly different. Data are presented as means±SEM.

Analysis of Sarcomeric Protein Phosphorylation by Pro-Q Diamond Phosphoprotein Gel Stain

Pro-Q Diamond (Invitrogen) gel stain was used to detect changes in phosphorylation states of the proteins. Myofibrils were prepared from DOCA-salt and sham models of the mice hearts, and pellets were solubilized in a non-reducing 2× Laemmli buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris HCl pH 6.8) (Reference 21). 25 mM N-ethylmaleimide (NEM) was added to the standard rigor buffer with Trition X-100, the standard rigor wash buffer and the 2× Laemmli buffer. An RC-DC assay (Bio-Rad) was used to determine protein concentrations. Samples were diluted at 1:1 ratio in reducing sample buffer (8 M urea, 2 M thiourea, 0.05 M tris pH 6.8, 75 mM DTT, 3% SDS, and 0.05% bromophenol blue) (Reference 41) and approximately 10 pg of protein was loaded on to a 12% resolving 1D SDS-PAGE gel. (References 42 and 43). The gels were stained and destained with Pro-Q Diamond according to the manufacturer's recommendations prior to imaging with a Typhoon 9410 scanner (GE Healthcare). Coomassie R-250 staining was used to normalize protein load to both MLC1 and the whole lane. Optical density of the proteins was determined using ImageQuant TL (GE Healthcare) software and results were exported to Excel for further analysis.

Analysis of Sarcomeric Protein Phosphorylation by Western Immunoblotting

Myofibrils were prepared from DOCA-salt and sham mice hearts with or without BH4 treatment (Reference 21) and pellets were solubilized in a reducing 2× Laemmli buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, 75 mM DTT and 0.125 M Tris HCl pH 6.8). An RC-DC assay (Bio-Rad) was used to determine protein concentrations. Samples were diluted at 1:1 ratio in reducing sample buffer (8 M urea, 2 M thiourea, 0.05 M tris pH 6.8, 75 mM DTT, 3% SDS, and 0.05% bromophenol blue) (Reference 41). Approximately 10 μg of protein was applied on to a 12% resolving 1D SDS-PAGE gel (References 42 and 43) and transferred onto a 0.2 μm PVDF membrane. The blot was blocked in 5% nonfat dry milk for 1 h. Anti-phospho-ser282-MyBP-C rabbit polyclonal antibody antibody (ENZO) and MyBP-C rabbit antidoby (Santa Cruz) was used at 1:1000 dilution along with anti-rabbit HRP-conjugated secondary antibody (Sigma) at 1:100,000 dilution to detect serine 282 site specific phosphorylation of MyBP-C. Anti-phospho-ser23/24-cTn1 rabbit polyclonal antibody (Cell Signaling) was used at 1:1000. Coomassie R-250 staining was used to normalize protein load to both actin and the whole lane. Optical density of the bands was measured with Image J and exported to Excel for further analysis.

Results

Improvement in Diastolic Function with BH_4

Ten days after surgery, I employed echocardiography to characterize the diastolic dysfunction. Treatment with a BH_4

supplemental diet was begun on post-operative day 11, and echocardiography was repeated on postoperative day 18. The results can be seen in FIG. **1** and Table 1 (below). Seven days of BH_4 administration in sham and DOCA-salt mice did not affect LV ejection fraction (FIG. **1**A) or fractional shortening (FIG. **1**B).

Mitral Doppler flow was measured at comparable heart rates (~average 370 beats/min) in all mice (Reference 10). As I have reported in this model, mitral E velocity, A velocity, and the E/A ratio were not significantly changed in all groups (FIGS. **1**C and **1**F). Nevertheless, mitral tissue Doppler E' was significantly decreased in the DOCA-salt mice indicating a pseudo-normal diastolic dysfunction stage. The ratio of E'/A' was significantly decreased in DOCA-salt mice and restored with BH_4 treatment (DOCA-salt+BH_4, 1.12±1.10 vs. DOCA-salt, 0.74 ±0.05, P <0.05). The sham and sham+BH_4 groups did not show any significant differences in E'/A' (FIGS. **1**D and **1**G). The E/E' ratio, a measure of left atrial pressure, was significantly increased in DOCA-salt mice, and restored to the control level after BH_4 administration (FIG. **1**E, DOCA-salt +BH_4, 34.5±2.2 vs. DOCA-salt, 43.7±2.7, P <0.05).

Improvement in Cardiomyocyte Parameters of Relaxation with BH_4

To confirm diastolic relaxation impairment in the model, I isolated single myocytes from each group and measured sarcomeric contraction and relaxation function, as seen in FIG. **2** and Table 2 (below). Sarcomere length was shortened in DOCA-salt mice and restored after BH_4 treatment (FIGS. **2**A-D). Fractional shortening was not changed in all groups (FIG. **2**C). On the other hand, the relaxation constant (τ), 50% relengthening time were significantly increased in DOCA-salt mice and returned to their normal levels after BH_4 treatment (FIGS. **2**E-F). The reduced relaxation rate in DOCA-salt mice reverted to control levels with BH_4 treatment (FIGS. **2**G-H).

To determine whether increased diastolic tension could be explained by active cross-bridge cycling, I treated the myocytes with BDM, a non-competitive inhibitor of active force-generation (Reference 16). Treatment of isolated myocytes with BDM (10 mM) increased residual sarcomere length in the sham and DOCA-salt groups. Treatment of either group with BH_4 resulted in significant relaxation as measured by sarcomere length. After BH_4 treatment, BDM had no effect, suggesting that BH_4 facilitated cross-bridge dissociation (FIG. **21**).

Myofilament Properties Altered by BH_4

In order to assess the relation between myocardial diastolic dysfunction and changes in myofilament properties, I performed analysis of tension and ATPase activity in skinned fiber preparations (FIGS. **3** and **4**). My results indicate that tension cost (ΔATPase/Atension) of skinned fibers from the DOCA-salt group (6.5±0.2) was significantly (P<0.05) reduced compared to shams (8.5±0.3) demonstrating that a slowing of cross bridge kinetics was responsible for diastolic dysfunction. Tension cost in fibers from the DOCA-salt+BH_4 group was increased (7.4±0.4, P<0.05) compared to the DOCA-salt group to a level not significantly different from either sham group (FIG. **3**, and Table 3 - below).

Maximum ATPase rate was also significantly reduced in DOCA-salt mice. This was accompanied by modest changes in maximum tension, pCa_{50} for tension and ATPase rate. BH_4 treatment increased maximum tension and ATPase rates in both sham and DOCA-salt mice, again with modest changes in pCa_{50} and significant changes in tension cost that varied in DOCA-salt versus sham mice. (FIG. **4**, and Table 3-below).

Myosin Binding Protein C Post-Translational Modifications

In one set of experiments, I determined potential modifications in phosphorylation of myofilament proteins employing Pro-Q diamond phospho-protein gel stain. With BH_4 treatment in the DOCA-salt mice, there was a decrease in phosphorylation of major myofialment proteins, MyBP-C, TnT3, cTnT4, MLC2 and titin, and no change in cTnl phosphorylation. The decreases in phosphorylation would tend to slow cross-bridge kinetics or increase diastolic stiffness, and thus are not likely to account for the reversal of effects of DOCA-salt on diastolic dysfunction with BH4 treatment. To further test this conclusion, I determined the level of site-specific phosphorylation changes in both MyBP-C and cTnl (FIGS. **10**A-E). Phosphorylation at Ser282 of MyBP-C was decreased in myofilaments from hearts of DOCA-salt mice compared to shams. BH_4 further reduced phosphorylation of MyBP-C at Ser282 in myofilaments from DOCA-salt mice, but did not significantly alter phosphorylation at this residue in sham myofilaments. Phosphorylation of cTnl at Ser23/24 was significantly reduced in myofilaments from DOCA-salt mice compared to sham, but was not significantly changed by BH4 in either from DOCA-salt or sham mice. Decreases in phosphorylation of MyBP-C and Tnl, have been previously demonstrated to slow cross-bridge kinetics (References 23 and 24). Thus, one could speculate that the decrease in phosphorylation of these two proteins that I observe in hearts from DOCA-salt mice may contribute to the impaired relaxation. However, I did not observe a reversal of phosphorylation of these two proteins in the presence of BH4 when diastolic function had recovered. Therefore, my results indicate that while the lower phosphorylation MyBP-C and cTnl may play a role in diastolic dysfunction, changes in S-glutathionylation of MyBP-C correlates with changes in diastolic function mediated by BH_4 in this model.

In view of my earlier findings indicating an increase in MyBP-C S-glutathionylation in cardiac myofilaments from DOCA-salt mice, I determined whether the BH_4 diet could reverse this modification. Representative gels and plotted data normalized to total protein loadings are shown in FIG. **6**. MyBP-C glutathionylation was significantly increased in the DOCA-salt group compared to all other groups, which were not significantly different from each other. In the data shown in FIGS. **7**A-H, I plotted diastolic function parameters (E/E' or E'/A' ratio) as a function of normalized MyBP-C glutathionylation. As shown in FIGS. **7**A and **7**B, the E/E' ratio was significantly, positively correlated with MyBP-C glutathionylation (Slope=3.23±0.90, R^2=0.305, **P <0.01). Moreover, TDI E'/A' ratio was negatively correlated with MyBP-C glutathionylation (Slope=−0.08±0.02, R^2=0.257, **P<0.01). Myofilament tension cost was also inversely correlated with both MyBP-C glutathionylation and E/E' echocardiographic data. However, both of phosphorylation level of Tnl and MyBP-C were not significantly correlated with tension cost (FIGS. **7**G-H).

Discussion

Results presented here provide new understanding of the role of cardiac myofilaments in the pharmacology and therapeutic efficacy of BH_4 for the treatment of diastolic dysfunction induced by pressure-overload. Overall, my results indicate that hypertension-associated diastolic dysfunction in this model likely arises mainly from a reduction in cross-bridge turnover kinetics and that administration of BH_4 results in amelioration of diastolic dysfunction by speeding these kinetics. Although correlative, my results support the hypothesis that changes in cross-bridge kinetics correlate with MyBP-C S-glutathionylation and that this oxidative modification may be responsible for the changes in cardiac dynamics. To the best of my knowledge, the present study is the first to report that treatment with BH_4 reduces increased levels of MyBP-C S-glutathionylation. Therefore, this post-translational modification may serve as a novel marker useful for the identification and treatment of diastolic heart dysfunction. Unlike in my previous study with ranolazine for the treatment of diastolic dysfunction, BH_4 reversed the glutathionylation of MyBP-C, suggesting that these two drugs work on the same disorder by different mechanisms (Reference 12).

Apart from my previous study indicating that S-glutathionylation correlated with changes in diastolic dysfunction and in tension cost, there is considerable evidence that modifications of MyBP-C affect diastolic function. Mutations in MyBP-C are known to induce diastolic dysfunction (Reference 25). MyBP-C is also a substrate for multiple kinases, including protein kinase (PK)A, PKC, PKD, and CaMKII (Reference 26). MyBP-C and its dephosphorylation have been shown to be associated with end stage human heart failure (Reference 27). MyBP-C dephosphorylation has also been associated with its degradation (References 26, 28-30), thick filament disruption, and contractile dysfunction (References 26, 28, 30). Phosphorylation of MyBP-C by PKA accelerates cross-bridge turnover rates (Reference 26). Interestingly, a non-PKA-phosphorylatable truncated mutant of MyBP-C (AllP-:[t/t]) exhibited a dilated LV chamber diameter, increased septal thickness, and depressed systolic function. This model also exhibited significant diastolic dysfunction because of slower cross-bridge cycling in the absence of baseline phosphorylation of MyBP-C (Reference 31). In general, my results fit with data in these studies indicating that effects of post-translational modifications in MyBP-C may be more prominently involved in altered cross-bridge kinetics and muscle dynamics than alterations in Ca-sensitivity. For example, employing loss of function models, Stelzer et al. reported that, in the intact myocardium, PKA phosphorylation of MyBP-C was a more prominent determinant of contraction and relaxation kinetics than phosphorylation of cardiac troponin 1 (cTnl), which was a more prominent determinant of Ca-sensitivity (Reference 32).

Nevertheless, in my experiments, MyBP-C phosphorylation did not correlate with diastolic dysfunction or BH_4 efficacy. In fact, compared to DOCA-salt myofilaments, the myofilaments from the DOCA-BH_4 treated hearts had reduced phosphorylation of MyBP-C as well as TnT, and MLC2. Yet BH_4 did not affect the phosphorylation of these proteins in the shams. A limitation of the study is that Pro-Q analysis measures total phosphorylation of a given protein, and MyBP-C contains multiple phosphorylation sites, the function of which are poorly understood. Thus, although I cannot exclude that site-specific phosphorylation may have contributed to diastolic dysfunction or the effect of BH_4, overall my data indicate that phosphorylation is not likely to contribute substantially to my findings of decreased tension cost and cross-bridge kinetics in the DOCA-salt myofilaments or to the amelioration of this effect with BH_4 treatment.

In addition to altered cross-bridge kinetics as a potential mechanism of diastolic dysfunction, modifications in sarcomeric diastolic function may be significantly affected by modifications in titin (References 33 and 34). In view of the potential modulation of extensibility by titin phosphorylation by protein kinase G (References 33 and 35), NO has been suggested to play an important role in regulating diastolic tone and ventricular filling through a cGMP-PKC dependent pathway (Reference 36). Moreover, PKG activation has been suggested to affect the reduction of Ca^{2+} sensitivity through Tnl phosphorylation at Ser23/24 and an increase in cross-bridge cycling rate, leading to acceleration of relaxation (References 37 and 38). However, in the present study, both titin and Tnl phosphorylation were not changed by BH_4 treatment in DOCA-salt mice suggesting another mechanism may be involved in the relaxation improvement via BH_4 in this model.

An important issue is the molecular mechanism of the effect of S-glutathionylation on MyBP-C function. Possible mechanisms are couched in terms of current hypotheses as to how MyBP-C controls cross-bridge kinetics. One plausible mechanism is that the radial disposition of MyBP-C in relation to the thick filament proper is a determinant of the rates of entry of the cross-bridges into and out of the cross-bridge cycle. Proximity of cross-bridges has been demonstrated to be increased by PKA-dependent phosphorylation (Reference 39). There is also evidence that MyBP-C directly interacts with actin in the thin filaments, and it is also plausible that modulation of thin filaments may result in increased cross-bridge kinetics (Reference 40). Whatever the case, my data indicate that modification of one or more cysteine residues of MyBP-C under oxidative control by S-glutathionylation is likely to alter the proximity of the cross-bridges to or their interactions with the thin filament. In the case of the DOCA-salt model, the modification is maladaptive and induces a diastolic abnormality. It is interesting to speculate that oxidative modification of MyBP-C may also serve as an adaptive mechanism in homeostasis, which modulates cardiac relaxation reserve by controlling cross-bridge kinetics.

In summary, hypertension-induced diastolic dysfunction was characterized by reduced cross-bridge kinetics and tension cost that was reversed by BH_4. The effect of BH_4 correlated with glutathionylation of MyBP-C, suggesting that this post-translational modification may lead to diastolic dysfunction and that BH_4 treatment may work by preventing this oxidative modification.

EXAMPLE II

Previously, I demonstrated that the depletion of NO bioavailability caused by increased reactive oxygen species (ROS) induced diastolic dysfunction with preserved systolic function through nitric oxide synthase (NOS) uncoupling in the heart. Depletion of tetrahydrobiopterin (BH4) causes NOS uncoupling, resulting in relaxation impairment of the heart. Mitochondria are one of the major cardiac oxidative stress sources, and manganese superoxide dismutase (MnSOD) is a mitochondrial antioxidant enzyme. In the present study, I sought to determine whether heterozygous knockout of the MnSOD gene (Sod2+/−) would be associated with diastolic dysfunction (DD) that could be ameliorated by BH4.

Methods

Echocardiography was used to determine DD in heterozygous MnSOD knockout mice. The mitral annulus longitudinal velocities (E', and A') were determined by pulsed-wave tissue Doppler from the apical four-chamber view. Mitochondrial ROS were measured by confocal microscopy and flow cytometry from isolated cardiomyocytes using MitoSOX Red. NO was measured by DAF-FM and by the Griess reaction. Contraction and relaxation impairment were assessed by lonOptix System.

Results

Mitochondrial ROS were elevated by 2.6-fold and NO level was reduced by 0.77-fold in cardiomyocytes from MnSOD deficient mice. The ratio of mitral annulus longitudinal velocities (E'/A') were significantly reduced indicating DD at MnSOD deficient mice (MnSOD 0.88±0.14 vs. WT 1.11±0.11). Resting sarcomere length was significantly reduced in MnSOD deficient cardiomyocytes compared to WT (MnSOD 1.68±0.01 μm vs. WT 1.84±0.01 μm, $P<0.001$) and the relaxation constant (tau) was significantly increased (MnSOD 0.12±0.01 vs. WT 0.09±0.01, $P<0.05$). BH4 treatment improved resting sarcomere length (1.77±0.01 μm, $P<0.001$) and tau (0.73±0.01, $P<0.001$) compared to MnSOD.

Conclusions

MnSOD deficiency was associated with impaired cardiac relaxation indicating DD, which could be improved with BH4 treatment.

TABLE 1

Transthoracic Echocardiography Measurements
In Vivo BH_4 Treatment in DOCA-Salt Mice

	Sham	Sham + BH_4	DOCA-salt	DOCA-salt + BH_4
LV M-Mode Protocol				
EF (%)	56.3 ± 3.9	51.1 ± 2.8	51.9 ± 4.4	54.5 ± 5.0
FS (%)	28.9 ± 2.8	24.7 ± 1.6	26.4 ± 2.7	26.6 ± 2.9
LVESD (mm)	2.74 ± 0.16	3.01 ± 0.11	2.86 ± 0.18	2.65 ± 0.21
LVEDD (mm)	3.86 ± 0.08	4.05 ± 0.10†	3.87 ± 0.11	3.67 ± 0.14†
Mitral Valve Protocol				
MV E (mm/s)	698.2 ± 35.9^	738.1 ± 31.1†&	613.7 ± 45.8&	555.0 ± 35.1†^
MV A (mm/s)	326.8 ± 30.1	357.5 ± 31.7	329.2 ± 48.7	367.8 ± 48.3
MV E/A ratio	2.31 ± 0.31	2.28 ± 0.33	2.15 ± 0.46	1.71 ± 0.33
Tissue Doppler Protocol				
E' (mm/s)	22.3 ± 1.7^*	24.4 ± 2.2†&	14.3 ± 0.8&*	16.8 ± 1.3†^
A' (mm/s)	19.4 ± 1.9	21.1 ± 1.9†	20.1 ± 1.4‡	15.2 ± 1.0†‡
E'/A' ratio	1.20 ± 0.09*	1.17 ± 0.06&	0.74 ± 0.05‡*&	1.12 ± 0.10‡
E/E' ratio	30.38 ± 1.17*	34.91 ± 6.81&	43.69 ± 2.73*&‡	34.53 ± 2.22‡
Sm	20.8 ± 1.8	22.9 ± 1.5†	18.5 ± 1.6	15.8 ± 1.5†

EF, ejection fraction;
FS, fractional shortening;
LVESD, left ventricle end systolic diameter;
LVEDD, left ventricle end diastolic diameter;
MV, mitral valve;
MV E, mitral inflow velocity peak early filing;
MV A, mitral inflow velocity peak late filing;
E', mitral annulus longitudinal velocity tissue Doppler early filing rate;
A' mitral annulus longitudinal velocity tissue Doppler late filing rate;
Sm, mitral annulus longitudinal velocity tissue Doppler systolic velocity. Data are represented as mean ± SEM (n = 7-9 per group).
#P < 0.05 for Sham vs. Sham + BH_4.
*P < 0.05 for Sham vs. DOCA-salt.
†P < 0.05 for Sham + BH_4 vs. DOCA-salt + BH_4.
‡P < 0.05 for DOCA-salt vs. DOCA-salt + BH_4.
^P < 0.05 for Sham vs. DOCA-salt + BH_4.
&P < 0.05 for Sham + BH_4 vs. DOCA-salt.

TABLE 2

Isolated Myocyte Contraction And Relaxation Parameters

	Sham	Sham + BH_4	DOCA-salt	DOCA-salt + BH_4
Diastolic SL, μm	1.78 ± 0.01*#	1.80 ± 0.01#†&	1.70 ± 0.01*&‡	1.77 ± 0.01†‡
Systolic SL, μm	1.59 ± 0.01*	1.61 ± 0.01&	1.54 ± 0.01*&‡	1.60 ± 0.01‡
Sarcomere shortening, %	9.47 ± 0.41	10.64 ± 0.61&	9.14 ± 0.44&	9.48 ± 0.49
Shortening velocity, μm/s	−2.38 ± 0.11#	−2.90 ± 0.16#†&	−2.03 ± 0.12&‡	−2.42 ± 0.14†‡
Time to peak shortening, ms	147.1 ± 5.2*	133.6 ± 0.3&	185.7 ± 6.8*&‡	146.2 ± 0.3‡
Time to 50% shortening, ms	47.3 ± 1.0*	45.6 ± 0.8	50.9 ± 1.3*‡	47.5 ± 0.7‡
Time to 90% shortening, ms	96.6 ± 2.4*#	87.3 ± 1.8&#	111.2 ± 3.6*&‡	93.5 ± 1.8‡
Relengthening velocity, μm/s	2.09 ± 0.11*#	2.75 ± 0.18#†&	1.60 ± 0.15*&‡	2.39 ± 0.18†‡
Time to 50% relengthening, ms	271.8 ± 10.9*#	204.7 ± 6.0&#	348.7 ± 15.4*&‡	240.3 ± 7.5‡
Time to 90% relengthening, ms	258.4 ± 26.0*#	258.7 ± 11.7&#	444.5 ± 19.6*&‡	300.0 ± 13.4‡
Relaxation constant, τ	0.09 ± 0.01*	0.07 ± 0.00&	0.28 ± 0.02*&‡	0.08 ± 0.01‡
No. of mice/cells	6/79	4/43	5/85	4/57

SL, sarcomere length. Measurements were performed under 1.0-Hz electrical stimulation, 10 V, in the presence of external Ca^{2+} of 1.2 mmol/L. Data are mean ± SEM.
N = 43-85 cardiomyocytes from 4-6 mice per group.
#P < 0.05 for Sham vs. Sham + BH_4.
*P < 0.05 for Sham vs. DOCA-salt.
†P < 0.05 for Sham + BH_4 vs. DOCA-salt + BH_4.
‡P < 0.05 for DOCA-salt vs. DOCA-salt + BH_4.
^P < 0.05 for Sham vs. DOCA-salt + BH_4.
&P < 0.05 for Sham + BH_4 vs. DOCA-salt.

TABLE 3

Effect Of BH_4 On Tension And ATPase Rate of Skinned Fiber Bundles	Sham	Sham + BH_4	DOCA-salt	DOCA-salt + BH_4
Maximum ATPase (pmol * s−1 * mg−1)	197.8 ± 2.3#*^	256.0 ± 1.7#†	177.5 ± 2.0*‡	296.0 ± 4.7†‡^
pCa50 for Tension	5.739 ± 0.006*^	5.753 ± 0.004	5.776 ± 0.004*	5.766 ± 0.005^
Maximum Tension (mN/mm^2)	22.22 ± 0.19#*^	30.43 ± 0.19#†	24.92 ± 0.15*‡	33.04 ± 0.25†‡^
Tension Cost ΔATPase/ΔTension	8.5 ± 0.3*	7.9 ± 0.3	6.5 ± 0.2*‡	7.4 ± 0.4‡

Data are means ± SEM.
N = 9-17 fibers,
#p < 0.05 for Sham vs. Sham + BH_4.
*P < 0.05 for Sham vs. DOCA-salt.
†p < 0.05 for Sham + BH_4 vs. DOCA-salt + BH_4.
‡P < 0.05 for DOCA-salt vs. DOCA-salt + BH_4.
^P < 0.05 for Sham vs. DOCA-salt + BH_4.

While this invention has been described as having preferred sequences, ranges, steps, order of steps, materials, structures, shapes, configurations, features, components, or designs, it is understood that it is capable of further modifications, uses and/or adaptations of the invention following in general the principle of the invention, and including such departures from the present disclosure as those come within the known or customary practice in the art to which the invention pertains, and as may be applied to the central features hereinbefore set forth, and fall within the scope of the invention and of the limits of the appended claims.

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The following references, and those cited in the disclosure herein, are hereby incorporated herein in their entirety by reference.

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What is claimed is:

1. A method of treating, reversing, or ameliorating diastolic dysfunction in a host with normal systolic function, comprising:

a) providing a host with normal systolic function with an ejection fraction of 50% or greater, and in need of treating, reversing, or ameliorating diastolic dysfunction; and

b) reducing S-glutathionylated myosin binding protein-C (MyBP-C) level by administering to the host a therapeutically effective amount of tetrahydrobiopterin (BH_4).

2. The method of claim **1**, wherein the step of administering comprises:

administering BH_4 in at least one form selected from the group consisting of a dietary supplement, a composition, a pharmaceutical composition, and a combination thereof.

3. The method of claim **1**, wherein the step of administering comprises:

administering BH_4 orally.

4. The method of claim **1**, wherein the host is a human.

5. The method of claim **1**, wherein the host is an animal.

6. A method of treating, preventing, reversing, or ameliorating diastolic dysfunction, comprising: modulating post-translational modification of myosin binding protein-C (MyBP-C) level by administering to a host in need thereof a therapeutically effective amount of tetrahydrobiopterin (BH4).

7. The method of claim **6**, wherein the step of administering comprises: administering BH4 in at least one form selected from the group consisting of a dietary supplement, a composition, a pharmaceutical composition, and a combination thereof.

8. The method of claim **6**, wherein the step of administering comprises: administering BH4 orally.

9. The method of claim **6**, wherein the host is a human.

10. The method of claim **6**, wherein the host is an animal.

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